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Phenolic deposits and tannin in the leaves of five xerophytic species from southern Africa

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Abstract. This paper reports on the histological distribution and structure of phenolic deposits in the leaves of *Diospyros ramulosa* (E. Mey. ex. A. DC.) De Winter, *Eriocephalus ericoides* (L. f.) Druce, *Galenia africana* L., *Hermannia disermifolia* Jacq. and *Rhus burchelli* Sond. ex Engl.. The tannin content of the leaves of these species was also determined in an attempt to ascertain whether the amount of phenolic deposits observed microscopically can be related to the quantitatively determined concentration. Results indicate that relatively large amounts of phenolic deposits, comprising different morphological types, occur in the leaves of four of the five species investigated. Furthermore, the levels of tannins determined quantitatively suggest that the positively stained cells contain molecules of a tanniniferous nature.

Key words: Anatomy; Histochemistry; Palatability; Phenolic deposits; Tannin; Xerophytic plants.

Introduction

A prominent but little understood feature of xerophytes is the accumulation of brownish material within the cells of the leaves. These materials are usually referred to as "tannins" or tanniniferous substances and are therefore presumed to be phenolic in nature. Plant phenolics constitute a group of natural products of structural diversity and wide phylogenetic distribution, of which the precise physiological function remains largely unknown (Zucker, 1983). Chaffe and Durzan (1973) mention that phenolic substances can be found in different cellular sites, for example, within the cell wall, in cytoplasmic vacuoles, or dispersed in the cytoplasm. Phenolic deposits are more often found in external tissues of plant organs, such as the epidermal layer, and have also been detected in relatively young and undifferentiated tissue (Salatino *et al.*, 1988). With regard to plant parts that normally bear cells with phenolic deposits, Esau (1977) comments that no tissue is entirely devoid of tannins.

This survey is aimed at determining the histological distribution and structure of phenolic deposits in leaves of five xerophytic species, which form an important part of the vegetation and play a prominent role in the vegetation dynamic processes of the arid and semi-arid regions of southern Africa. The tannin concentration in the leaves of these species was also determined in an attempt to ascertain if the amount of phenolic deposits observed microscopically can be related to the quantitatively determined concentration.

Materials and Methods

Leaves of *Diospyros ramulosa* (E. Mey. ex A. DC.) De Winter, *Eriocephalus ericoides* (L. f.) Druce, *Galenia africana* L., *Hermannia disermifolia* Jacq. and *Rhus burchelli* Sond. ex Engl. were collected in the arid Namaqualand region of southern Africa. This is a win-

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ter rainfall region with a long term average rainfall of 127 mm per annum. Rainfall is however, erratic and seasonal droughts occur frequently. The temperature varies from -1°C to 48°C.

Histological Study of Phenolics

Leaf phenolic content was determined by the histochemical localization of phenolics. Material was fixed in 4% paraformaldehyde at pH 7.2 and dehydrated in a alcohol series (O'Brien and McCully, 1981). Segments were embedded in 2-hydroxyethyl methacrylate according to the GMA-method of O'Brien and McCully (1981) and sectioned at 3 μm. The presence of phenolic inclusions were indicated by staining sections with $FeCl_3$ (Johansen, 1940) and confirmed with the nitroso -reaction as suggested by Reeve (1951). For general histology, sections were stained with 0.05% aqueous toluidine blue O (Theunissen, 1990). Micrographs were taken with a Zeiss photo microscope.

Tannin Analysis

A protein precipitation assay developed by Hagerman (1987) was used for determining the amount of tannin present in the leaf tissue of the species used in this study. In the assay tannin diffuses through a protein -containing gel, whereby a visible disk-shaped precipitate develops as the tannin interacts with the protein. This is a simple and sensitive method specific for tannin, in that non-tannic substances such as flavonoids, benzoic acids or hydroxycinamic acids do not interfere with the assay.

Leaves were dried at 30°C for 48 h, cut into small pieces ($\approx$5 mm^2) and ground with a mortar and pestle. The powder was extracted for an hour at room temperature with 70% (w/v) aqueous acetone (Hagerman, 1988), using a solvent-tissue ratio of 0.5 ml solvent per 100 mg tissue. The extracts were assayed using the radial diffusion assay (Hagerman, 1987). Wells were made in the bovine serum albumin-containing plates with a 4 mm punch, and three 8 μl aliquots of extract were applied to each well with a Gilson micro-pipette. After an incubation period of 72 h, the diameter of the ring which developed was measured. The diameter squared is proportional to the amount of tannin added to each well (Hagerman, 1987). The amount of tannin precipitated was determined from a standard curve run with purified tannic acid (obtained from Merck, Art. 773) and expressed as milligrams tannin per milligram dry tissue.

Results

Histological Localization of Phenolic Deposits

In the leaves of *Hermannia disermifolia* phenolic deposits occur in the mesophyll cells, bundle sheath extensions of the main and secondary veins, parenchyma cells of the xylem and phloem, and epidermal cells (Fig. 1a). Phenolic substances, which are not abundant in the mesophyll cells of this species, occur mainly as dark brown amorphous material which partially or completely fills the cell lumen (Fig. 1b). Phenolic compounds also occur as small, light brown globules dispersed throughout the cytoplasm of the mesophyll cells or as granular material which completely fills the cell lumen (Fig. 1d). Collenchyma cells of the bundle sheath extensions contain phenolic substances which fill the cell lumen but in appearance are similar to the former mentioned (Fig. 1a).

The epidermal layers of *H. disermifolia* contain numerous phenolic inclusions comprising four structurally dissimilar types. The most common types consist of dark brown amorphous (Fig. 1b) or granular (Fig. 1e) material filling the cell lumen. Fine granular phenolic substances which stain blue with toluidine blue O, fill the cell lumen of the epidermal cells (Fig. 1e). Spherical globules dispersed thoughout the cytoplasm were also observed in the epidermal layers of this species (Fig. 1d).

Phenolic deposits commonly occur in the mucilage cells which are characteristic of the epidemal layer of *H. disermifolia*. These light brown amorphous substances are situated adjacent to the outer periclinal wall of the epidermal cells, thereby filling the reduced cell lumen after the inner periclinal wall has become mucilaginous (Fig. 1f). Dark brown tanniniferous substances also occur in the stalk cells of the stellate trichomes (Fig. 1c), while small amounts of light brown phenolic globules are present in the head cells of glandular trichomes (Fig. 1g).

The leaves of *Rhus burchelli* contain phenolic substances in the mesophyll cells, parenchymatous bundle sheaths, bundle sheath extensions of the main and secondary veins, phloem parenchyma cells of the main vein, and in the epidermal cells (Fig. 2a).

These substances are abundant in the mesophyll cells of *R. burchelli* and consist of dark or light brown

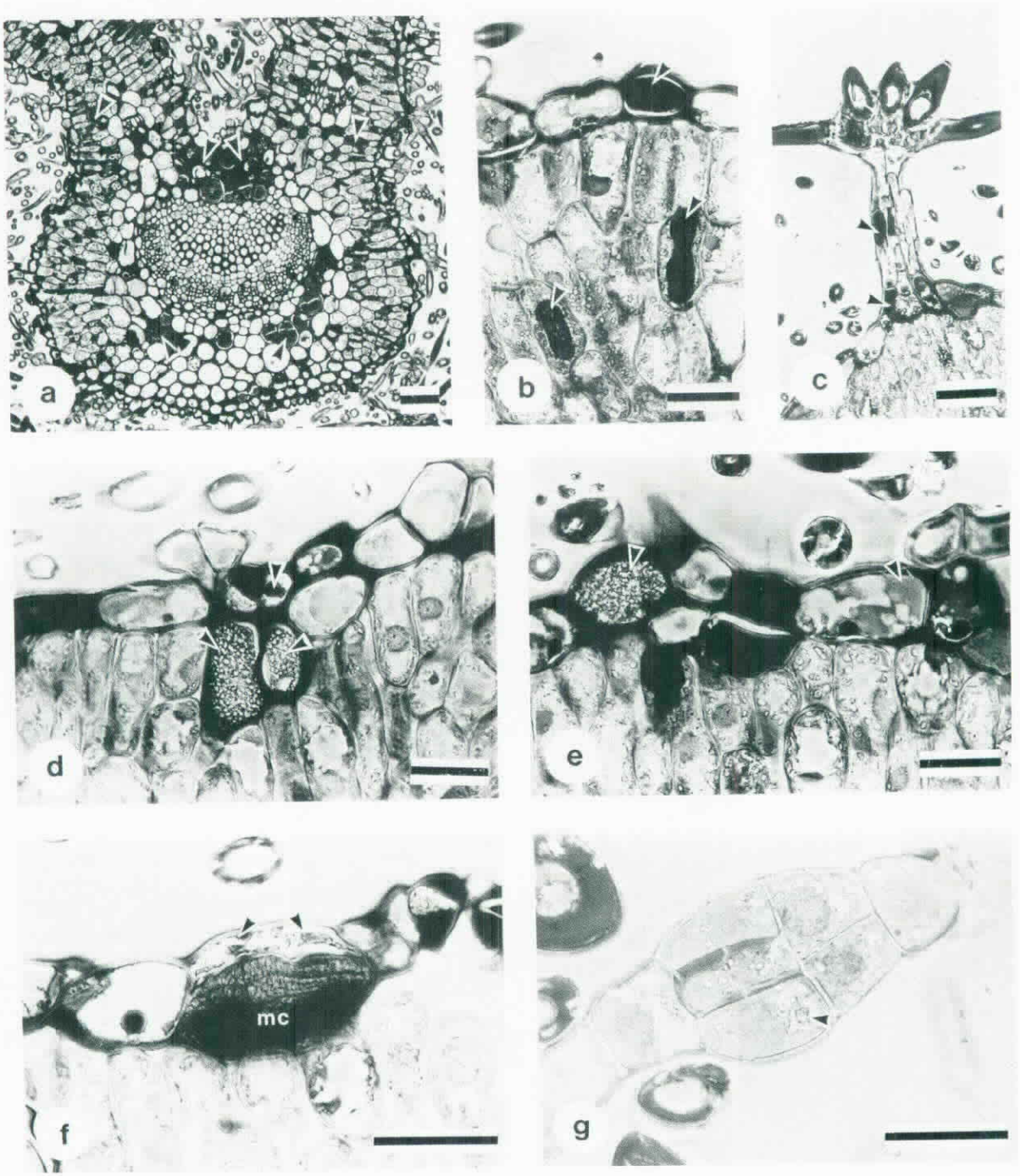

Fig. 1a. Light micrograph showing the distribution of phenolic deposits in the leaves of *Hermannia disermifolia* as seen in cross section stained with toluidine blue O. Bar = 10 μm.

Fig. 1b–g. Phase contrase light micrographs of the leaves of *Hermannia disermifolia* as seen in cross section stained with toluidine blue O showing: (b) Dark stained amorphous material in the epidermal and mesophyll cells. (c) A stellate trichome with dark stained amorphous material in the stalk cells. (d) Granular phenolic deposits in the mesophyll cells and darkly stained globules in the epidermal cells. (e) Dark stained granular phenolic deposits and phenolic deposits which stain blue with toluidine blue O in the epidermal cells. (f) A mucilage cell (mc), containing phenolic deposits in the cell lumen. (g) Head cells of a glandular trichome containing small amounts of phenolic material. Bars = 5 μm.

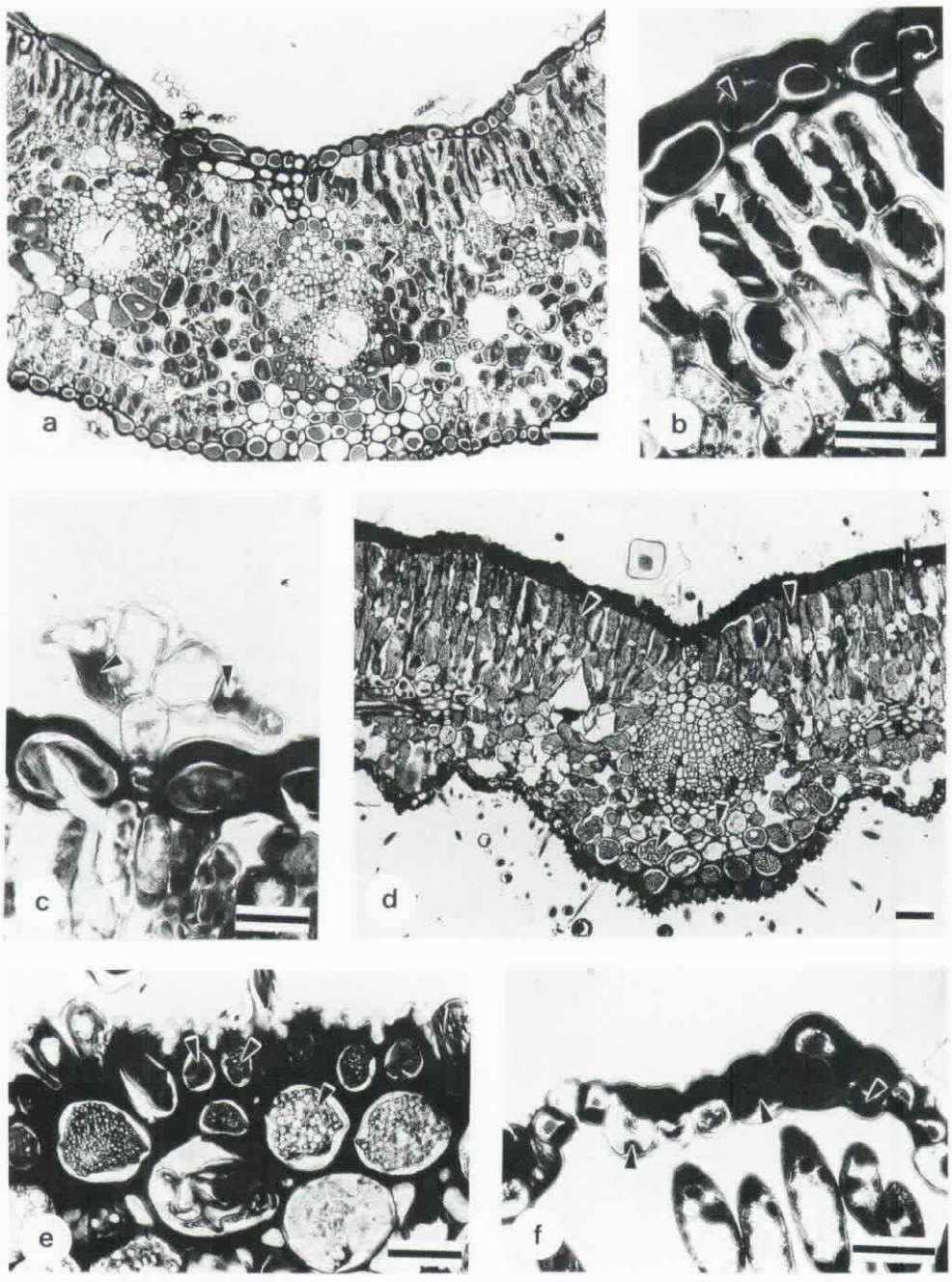

Fig. 2a. Light micrograph showing the distribution of phenolic deposits in the leaves of *Rhus burchelli* as seen in cross section stained with toluidine blue O. Bar = 10 μm.

Fig. 2b-c. Phase contrast light micrographs of the leaves of *Rhus burchelli* as seen in cross section stained with toluidine blue O showing: (b) Dark stained amorphous material in the epidermal and mesophyll cells. (c) A glandular trichome containing dark stained amorphous material in the head cells. Bars = 5 μm.

Fig. 2d-e. Leaves of *Diospyros ramulosa* as seen in cross section stained with toluidine blue O. (d) Light micrograph showing the distribution of phenolic deposits. Bar = 10 μm. (e) Phase contrast lightmicrograph showing dark stained amorphous and granular phenolic deposits in the epidermal cells and collenchyma cells of the bundle sheath extensions. Bar = 5 μm.

Fig. 2f. Phase constrast lightmicrogtaph of the leaf epidermal and mesophyll cells of *Eriocephalus ericoides* as seen in cross section stained with toluidine blue O showing phenolic deposits in the epidermal cells. Bar = 5 μm.

amorphous material which completely fills the cell lumen (Figs. 2a and 2b). Phenolic inclusions which occur in the bundle sheaths, bundle sheath extensions and phloem parenchyma cells have an amorphous appearance (Fig. 2a) and stain blue with toluidine blue O. In the bundle sheath extensions however, these substances may also have a granular appearance. Tanniniferous substances in the epidermal layers of this species, which stain a variety of colours with toluidine blue O, namely, light blue, dark blue or green, have an amorphous structure and in most cases completely fill the cell lumen (Fig. 2b). Dark brown, amorphous material also occurs in the head cells of glandular trichomes (Fig. 2c).

It is perhaps significant to mention that a certain amount of the substances in the leaf tissue of *R. burchelli* may not be phenolics, but may in fact be resin, which is are terpenoid compounds found in the leaves of most members of the Anacardiaceae (Metcalfe and Chalk, 1950). However, no histochemical reagent is available to detect resinous matter accurately in plant tissues. Since some resin components are lipophilic in nature, lipid stains such as nile blue may be used as an indication of the presence of resinous substances (Vermeer and Peterson, 1979).

The leaves of *D. ramulosa* contain large amounts of phenolic deposits which fill the cell lumen of the epidermal, mesophyll and collenchyma cells (Figs. 2d and 2e). Results indicate that phenolic deposits are more prominent in the mesophyll and epidermal cells of young leaves than in older leaves. These deposits have an amorphous or granular appearance in both young and old leaves (Fig. 2e).

In *E. ericiodes* phenolic deposits mainly occur in the epidermal layers. These substances appear as light or dark brown globules situated on the periphery of the epidermal cells, or completely filling the cell lumen (Fig. 2f).

No phenolic inclusions were observed in any cells of the leaves of *Galenia africana*.

Tannin Analysis

The leaves of *D. ramulosa* have the highest levels of tannin, followed by *E. ericoides* (Table 1). The levels of tannin in *R. burchelli* were slightly lower than those of *H. disermifolia*. However, tannin was absent in the leaves of *G. africana* (Table 1).

Table 1. *Tannin concentrations in the leaves of five xerophytic species*

Species	Tannin[1] (mgtannin/100mg dry tissue)[2]
Diospyros ramulosa	0.35±0.02
Eriocephalus ericoides	0.23±0.03
Hermannia disermifolia	0.15±0.01
Rhus burchelli	0.12±0.02
Galenia africana	0.00±0.00

[1]Tannic acid (Merck, Art. 773) used as standard.
[2]Values shown are the means of three replicates (±SD).

Discussion

Quantitative analysis indicate that tannins occur in relatively large amounts in four of the five xerophytic species investigated. This can possibly be related to the unfavourable environmental conditions which these species have to tolerate, in that it has previously been determined that plants which grow under unfavourable environmental conditions, contain relatively large amounts of phenolics (Mckey *et al.*, 1978; Lowther *et al.*, 1987). Grieve (1953) examined the leaves of Australian sclerophyllous plants and came to the conclusion that the concentration of tanniniferous substances in these plants increase when drought conditions intensify. Furthermore, Theunissen (1990) and Theunissen and Jordaan (1990) have recently found that leaves of *Themeda triandra* Forssk. and *Eragrostis racemosa* (Thunb.) Steud. inhabiting dry areas have more phenolic deposits than plants occupying moist habitats.

Although phenolic deposits occur in relatively large amounts in the mesophyll tissue of *D. ramulosa* and *R. burchelli*, this study indicates that large amounts of phenolic substances are more often found in the epidermal layers of the four xerophytic species investigated. The function of protecting the underlying mesophyll cells against excess visible and/or ultraviolet radiation has been ascribed to tannins occuring in large amounts in epidermal cells (Salatino *et al.*, 1988). An increase in the formation of phenolic substances may also be the result of disrupted metabolism caused by high radiation levels which usually prevail in arid regions. Mole *et al.* (1988) have found that *Diospyros thomasii* Hutch. and Dalz. produces high levels of tannins when subjected to increased light radiation levels. This effect is ascribed to enhanced photosynthesis

which occurs under high light intensity, resulting in the formation of large amounts of carbohydrates. This in turn leads to an increased C:N ratio resulting in the production of more carbon based compounds, including phenolic substances.

The variety of morphological forms and the different staining reactions of the phenolic inclusions may indicate the presence of different types of phenolics in these species. The differences in staining reactions after staining with toluidine blue O, according to Ling-Lee *et al.* (1977) are indicative of different types of macromolecules, constituting these phenolics.

An antiherbivory function has been ascribed to tannins in previous studies (Swain, 1979; Robbins *et al.*, 1987a; 1987b), in that the protein precipitating action of tannins results in the ingested plant material being somewhat indigestible, unpalatable and of poor nutritional value (Bernays, 1978). However, this does not seem to be applicable to the species investigated here, as *D. ramulosa*, although containing high tannin levels, is considered to be a highly palatable species (Le Roux and Schelpe, 1988). *E. ericoides* which has relatively high levels of tannin is generally considered to be palatable, although this species may become unpalatable, depending on the season and the aridity of the region where it occurs (Le Roux and Schelpe, 1988). In contrast, *H. disermifolia* and *R. burchelli* which in turn contain lower levels of tannin than *D. ramulosa* and *E. ericoides*, are both highly unpalatable species (Le Roux and Schelpe, 1988). This unpalatability may however be ascribed to the thick layer of stellate trichomes covering both leaf surfaces of *H. disermifolia*, or to the resinous compounds in the leaves of *R. burchelli*. It is of interest that the leaves of *G. africana*, in which no tannin was detected, are avoided by herbivores. However, saponins which is a common substance in the leaves of this species (Le Roux and Schelpe, 1988), may play a significant role as a deterrent against herbivores.

Indications are that the amount of phenolic deposits observed microscopically can be related to the quantitatively determined tannin concentration. This is clearly indicated in *D. ramulosa*, in that this species has large amounts of phenolic deposits and a relatively high tannin content, whereas both phenolic deposits and tannin were absent in the leaves of *G. africana*. Furthermore, the quantitative analysis indicates that *R. burchelli* has low levels of tannin, which supports the possibility that a large amount of the inclusions present in the leaves of this species are resinous in nature.

From an ecological viewpoint this study emphasizes the necessity for further investigations especially with regard to the presence, distribution and function of phenolic compounds in xerophytic species which form an important part of natural pasture in southern Africa.

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南非五種旱生植物葉中酚類堆積及鞣酸含量

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本文報告酚類在 *Diospyros ramulosa* (E. Mey. ex A. DC.) De Winter, *Eriocephalus ericoides* (L.f.) Druce, *Galenia africana* L., *Hermannia disermifolia* Jacq. and *Rhus burchelli* Sond. ex Engl. 等植物葉中之堆積與分佈情形，並籍著測定這些植物葉中鞣酸含量，以便探討顯微鏡觀察下酚類之堆積量與實際濃度之關係。結果顯示五種植物中有四種葉中含有較大量酚類堆積，而且堆積形態不同。另外鞣酸定量結果顯示有酚類堆積的細胞含有鞣酸成份。